Morphogenesis of rod shaped sacculi

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Abstract 200 words

For growth and division of rod shaped bacteria, the cylindrical part of the sacculus has to be elongated and two new cell poles have to be synthesized. The elongation is performed by a protein complex, the elongase that inserts disaccharidepentapeptides at a limited number of discrete sites while using the cytoskeletal MreB helix as tracking devise. Upon initiation of cell division by positioning of the cytoskeletal Z-ring at mid cell, a switch from dispersed to concentrated local peptidoglycan synthesis occurs. From this point on,

peptidoglycan synthesis is for a large part redirected from elongating activity to synthesis of new cell poles by the divisome. The divisome might be envisioned as an extended elongase because apart from its basic peptidoglycan synthesis activity, specific functions have to be added. These are, the conversion from cylinder to sphere, the invagination of the outer membrane, and the adition of hydrolases that allow separation of the daughter cells. The elongase and the divisome are dynamic hyperstructures that probably share part of their proteins. Although this multifunctionality and flexibility form a barrier to the functional elucidation of its individual subunits, it helps the cells to survive a variety of emergency situations and to proliferate securely.

1. Introduction

The shape of bacteria is maintained by the shape of their peptidoglycan layer, as the isolated peptidoglycan bag or sacculus retains the morphology of the cell it is isolated from (Fig.1). Comprehension of the mechanism that allow bacteria to differentiate their shape into cell poles and straight cylinders, banana-shaped cylinders, spirals, and American footballs to mention a few variations [1], is therefore a prerequisite for the understanding of bacterial morphology. To unseal these mechanisms, it is not sufficient to understand all the enzymatic reactions involved in the biosynthetic pathway of the sacculus (This issues .....).

For the synthesis of a three-dimensional structure also the coordinates of synthesis and hydrolysis or its topography has to be included to answer the question of where the

peptidoglycan is synthesized. Topological information has to be added because the sacculus is embedded in the bacterial envelope that is separated from its metabolic compartment the cytoplasm. Lastly, the dimension, time, has to be implicated because of the time dependent nature of the bacterial cell cycle. This review will deal with the questions where, when and by which proteins the peptidoglycan layer synthesis takes place in rod shaped bacteria.

Due to the differential affinity of a variety of ß-lactams for penicillin-binding proteins (PBPs), one of the earliest observations has been that rod shaped bacteria could grow temporarily either as spheres or as filaments. As a consequence construction of the cylindrical part of the cell, which might be termed elongation, and of the cell poles, termed division, have become separate events in most studies. Therefore, we will discuss the two processes in this order, although this separation might not completely justified as will follow from this review. By default, the Gram-negative *Escherichia coli* will be the model organism for rod shaped bacteria and when necessary illustrative data from the Gram-positive *Bacillus subtilis* or the gram-negative *Caulobacter crescentus* will be used.

2. Proteins involved in elongation and their interaction

The biosynthetic reaction pathway to peptidoglycan occurs in three stages. The cytoplasmic stage takes care of the synthesis of the activated nucleotide cytoplasmic precursors UDP-*N*-acetyl-glucosamine (UDP-GlcNAc) and UDP-*N*-acetylmuramyl-pentapeptide (UDPMurNAc-pp) via an enzymatic reaction cascade described elsewhere

in this issue. This is followed by the membrane linked lipid cycle of reactions that translocates the lipid-linked β-(1,4) disaccharide peptidoglycan building-unit known as lipid II across the cytoplasmic membrane (This issue ....). In the periplasmic stage, these precursors are inserted into the peptidoglycan layer by the PBPs (This issue...). The cytoplasmic stage is thought to be identical for elongation and division of the bacterium. It is in the membrane that the first signs of discrimination of the two steps seem to occur. Proteins that cause the cell to grow as spheres when they are defect, such as MreB, MreC, MreD, RodA, and PBP 2, are either associated with the membrane or integrated in the membrane.

RodA is a 370 amino acids residues integral membrane protein of 40 kDa [2] that was discovered as a morphological mutant that caused the cells to grow as spheres [3]. Conditional mutants or deletions of homologues of RodA in *B. subtilis, Salmonella thyphimurium* and even in the ovoid coccus *Streptococcus thermophiles* result in spherical growth [4-6]. Because RodA in association with PBP2 seems to be essential for peptidoglycan synthesis in PBP1B-free membrane preparations of *E. coli* [7], it has been postulated that RodA could be involved in the translocation of the lipidII peptidoglycan precursors across the cytoplasmic membrane. Although, it has been established that proteins must be involved in the lipid-II translocation and that translocation seems to be coupled to the transglycosylation reaction of the PBPs or monofunctional transglycosylases [8], the identity of the protein(s) responsible for the translocation is still a mystery.

Mecillinam or amdinocillin (Fl1060, [9]) specifically inhibits the peptidoglycan transpeptidase **PBP2** [10, 11] causing spherical growth, a substantial increase in the

diameter of the cells and subsequently cell death. The latter can be prevented by overexpression of the cell division proteins FtsQAZ (See section 3) or by increasing the stringent response transcription regulator ppGpp [5, 12-14]. The interpretation is that PBP2 is essential for the maintenance of the diameter of the cells (see also section 5) and in its absence the diameter and therefore the volume of the cells increases uncontrolled. Consequently, the concentration of FtsZ becomes too low to initiate cell division and new polar peptidoglycan synthesis. The other rod shape determining RodA and Mre proteins are also not essential provided that the strains contain mutations that prevent the uncontrolled increase of their diameter. Such strains can be mecillinam resistant [15], overproduce ppGpp [5], overproduce cell division proteins [16, 17] or are osmoticaly protected [18]. Because PBP2 has transpeptidase activity only, it will have to associate with at least one class A PBPs with glycosyltransferase and transpeptidase activity and perhaps also a monofunctional glycosyltransferase. Based on *in vivo* crosslinking experiments some evidence exists that the majority of the PBPs are part of two different protein complexes [19].

MreB (originally EnvB) mutants that grew as spheres were discovered in 1969 by Normark [20] and in combination with **MreC** and **MreD** by Wachi et al., in 1987 [21, 22]. The fusion of GFP to the *B. subtilis* MreB protein in 2001 ([23] and in other species [24, 25], which showed that MreB (and its paralogues in *B. subtilis*, Mbl and MreBH) localizes as helix underneath the cytoplasmic membrane and the subsequent publication of its actin-like crystal structure [26] focused the attention of the scientific community on these proteins. MreB is a soluble cytoplasmic protein of 37-kDa that polymerizes in an ATP dependent fashion [26, 27]. MreC is a 40-kDa bitopic membrane protein with a

large periplasmic domain and MreD is an integral membrane protein of 19-kDa. Bacterial two-hybrid analysis [28] showed that MreC interacts with itself, presumably forming a dimer [29], and it interacts with MreD and with MreB [17]. MreB interacts with MreC but not with MreD (Fig. 2). The association of the three proteins is essential for lateral growth as depletion of each of these proteins is sufficient for spherical growth [17]. Interestingly, MreB is not able to localize in its helical pattern in spherical cells that are depleted of RodA, MreC, or MreD [17], but it localizes normally in cells that are spherical due to inhibition of the function of PBP2 by mecillinam [30]. Inhibition of MreB by its specific inhibitor A22 [31, 32] or inhibition of PBP2 by mecillinam results in a change from rod to sphere in the same time window [30] with identical changes in the muropeptide composition of the sacculus [33]. These changes are a significant shortening of the glycan chain length, a 60% decrease in pentapeptides, a 20% increase in crosslinking and an increase in the percentage of lipoprotein-containing muropeptides [33] This clearly points to a relationship between MreB and PBP2. PBP2 of *Caulobacter crescentus* localizes in a band like pattern that is dependent on the presence of MreB and MreC [25, 34, 35]. MreC itself was also shown to localize in a helical pattern in the periplasm [34, 36, 37]. Ramoplanin binds to the reducing ends of the nascent glycan chains [38, 39]. Application of a sub-inhibitory concentration of fluorescent ramoplanin or vancomycin to growing cells revealed a helical staining pattern along the side-walls of *B. subtilis* [40] of E. coli [33]. Together these data suggest the presence of a protein complex consisting of MreBCD, RodA and PBP2 that directs lateral peptidoglycan synthesis. The helical track provided by the MreBCD most likely ensures the disperse insertion of new peptidoglycan precursors into the existing lateral cell wall. Like actin,

MreB monomers treadmill through MreB filaments by preferential polymerization at one filament end and depolymerization at the other filaments end [41, 42]. It seems likely that this dynamic movement is passed on to the precursor inserting protein complex (see also section 4.1).

The lipid II peptidoglycan precursor that is synthesized by the membrane associated glycosyltransferase **MurG** (37.7 kDa) [43-45] is supposed to be the substrate of this elongase. It is, therefore, not surprising that in *E. coli* MurG could be co-immunoprecipitated with MreB and that the lateral localization pattern of MurG was lost in an MreCD deletion background [46]. Similarly, the integral membrane protein **MraY** (40 kDa) [47-49] that catalyzes the transfer of the phospho-MurNAc-pp motif of UDP-MurNac-pp to the lipid carrier undecaprenyl phosphate, to form the so-called lipid I peptidoglycan precursor to be used by MurG [50, 51], was shown to co-immunoprecipitate with MreB and MurG [46]. Most likely, MraY and MurG are part of the protein complex that inserts the peptidoglycan precursors into the peptidoglycan layer during elongation of the cells, and which therefore could be termed **elongase** (Fig. 2). In addition, the elongase should contain the lipid II translocase if the RodA protein does not provide this function. Alternatively, a combination of the integral membrane proteins might form the translocase (Fig 2B).

3. Proteins involved in cell division and their interactions

MurG and MraY are both essential proteins with no alternatives available in *E. coli*. Therefore, both proteins are the most likely candidates to provide lipid I and lipid II precursors for the synthesis of the new cell poles. In agreement with this notion MurG was found to localize as well-separated foci in the cylindrical part of the cell and as concentrated foci at the site of division in *E. coli* [46] and in *C. crescentus* [52]. The presence of the mid cell localization of MurG was only visible after assemblage of all cell division proteins at mid cell in *E. coli* [46] but coincided with the assemblage of the Z-ring in *C. cresentus* [52]. The observation in *E. coli* does not exclude a somewhat less abundant activity of MurG at the initiation of cell division that coincides with the assembly of the Z-ring.

3.1. Assembly of the Z-ring

The tubulin homologue FtsZ [53] is a GTPase that is conserved in most of bacteria. This abundant protein (~5000 copies per cell in *E. coli* [54] and den Blaauwen unpublished) polymerizes in a GTP dependent fashion. It is a highly dynamic structure. Subunits continuously exchange with a half-life of 8-9 s [55]. The Z-ring is built from overlapping segments of protofilaments of on averaging about 30 subunits (~120 nm in length) [56]. FtsZ outside the ring localizes in a helix-like pattern and moves rapidly within this pattern independently from MreB helix or MinD oscillation (see for a review on the min system [57]). Thus FtsZ not only forms the Z ring but also is part of a highly dynamic, potentially helical cytoskeleton [58, 59].

The formation of the Z-ring, which is the earliest step in cell division, requires the presence of FtsA or ZipA that tether FtsZ filaments to the membrane. They interact with a short conserved sequence of the C-terminal end of FtsZ [60-62]. ZipA binds to the membrane through an essential N-terminal transmembrane segment (TMS) that is linked to the FtsZ interacting C-terminal globular domain by an extended linker region [63]. FtsA is an actin structural homolog [64]. It binds to the membrane through a conserved C-terminal amphipathic helix that does not appear to be specific as it can substitute for that of MinD. FtsA is proposed to bind to the membrane before interacting with FtsZ, this interaction plays an essential role in cell division [65]. Cardiolipin-rich domains are present in the septal and on the polar membrane regions of *E. coli* [66, 67]. The amphipathic helix of FtsA has basic residues on its hydrophilic side and might interact with acidic cardiolipin (CL) in the septal region analogous to the polar assembly of MinD, which is promoted by CL [68]. Interestingly, overexpression of MurG increased the amount of CL per cell 7 fold [69]. This could imply that the MurG attracts CL at mid cell.

A conserved region in subdomain 2B of FtsA responsible for interaction with FtsZ has been identified by isolation of FtsA mutants that are able to bind to the membrane but fail to interact with FtsZ [62]. FtsA* contains a modification of R286 into W in subdomain 2B and can bypass the requirement for ZipA in the assembly of the Z-ring and its functioning in cell division by stabilizing the FtsZ ring [70, 71]. Subdomain 1C of FtsA is involved in the recruitment of the other components of the divisome [72].

Two non related intracellular nonessential proteins (ZapA in *E. coli* [73, 74] and EzrA in *B. subtilis* [75, 76]) appear to affect the stability of the Z-ring. A proper ratio

between FtsZ to FtsA or ZipA is required for assembly and maintenance of the Z-ring. FtsE/X is an ABC transporter combination of an integral membrane protein FtsX and a cytosolic membrane associated protein FtsE [77]. The complex interacts with FtsZ [78] and allows the cell to assemble the Z-ring under conditions of high osmolarity [79].

3.2 Maturation of the divisome.

The Z-ring is required for the localization of other cell division proteins, FtsK, FtsQ, FtsL, FtsB, FtsW, PBP3, (also called FtsI), and FtsN to form the divisome (also called septosome) at mid cell. All these proteins localise in a sequential and interdependent manner at the division site (Fig. 3). Proteins FtsK-FtsN are recruited almost simultaneously at the division site approximately 17 min after the assembly of the Z-ring [80, 81]. A mutant that underexpresses S-adenosylmethionine synthase forms filaments without visible septa in which FtsQ, FtsW, PBP3 or FtsN fail to assemble to the Z-ring. This result suggests that some methylation is required before the complete divisome can be assembled [82]. MraW also called YabC might be the methylase. Interestingly, the gene *mraW* with unknown function is located in the dcw cell division gene cluster upstream from *ftsL* [83].

FtsK is a multifunctional and multidomain protein. The cytoplasmic C-terminal domain forms the translocation machine, which is involved in chromosome segregation [84]. The N-terminal domain is formed of 4 transmembrane segments and is required for cell division [85]. Expression of *ftsA(R286W)* or overexpression of *ftsQ* allows the cell with a complete deletion of *ftsK* to survive and divide, although many of these *ftsK* null cells formed multiseptate chains [70]. The cytoplasmic and transmembrane domains of

FtsQ, are sufficient to confer viability to *ftsK* null cells. These data suggest that FtsK is involved in stability of the cell division machine and has a role in the closure of the pole. Deletion of *dacA* encoding PBP5/DD-carboxypeptidase can suppress the *ftsK*44(ts) phenotype which is due to a modification of G80 into A in a transmembrane segment. This modification might affect the conformation of a periplasmic loop of FtsK in contact with peptidoglycan [86].

FtsQ, **FtsL** and **FtsB** are bitopic membrane proteins with a small N-terminal intracellular region, a transmembrane segment and a larger periplasmic domain. The structure of the extracellular region of *Geobacillus stearothermophillus* FtsQ comprises three domains [87]. The N-terminal α domain corresponds to the POTRA domain predicted on the basis of bioinformatics analysis and is proposed to function as a molecular chaperone [88]. The N-terminal region and α domain seem to act cooperatively to bring FtsQ to midcell [89]. The β domain has a unique 3D fold and might modulate interactions of the flanking domains. The C-terminal γ domain is sensitive to proteases and unstructured in the absence of division proteins and corresponds to the region that interacts with FtsL and FtsB in *E. coli* [44]. FtsQ interacts with FtsL, FtsB, PBP3, FtsW and FtsN as shown by a two-hybrid system and co-immunoprecipitations [90-92]. Overproduction of FtsQ inhibits division of cells producing FtsZ, FtsA or PBP3 mutants. These data suggest that FtsQ could play a role in regulating new pole synthesis [93, 94].

FtsL and FtsB seem to interact in a coiled-coil structure through their periplasmic domain. [95]. They are dependent on each other for proper localization and depend on FtsQ for their localization at the division site. A fusion of FtsQ to ZapA is recruited to the

Z-ring independently of FtsA and directs the recruitment of FtsL, FtsB and PBP3. It can also back recruit FtsK indicating that both proteins can interact directly [96]. A complex of FtsL, FtsB and FtsQ can be co-immunoprecipitated in the absence of FtsK, FtsW and PBP3, which indicates that these proteins probably form a complex before assembling into the divisome [97].

Expression of ZapA-FtsL or ZapA-FtsB allow the recruitment of FtsW and PBP3 in the absence of FtsQ showing that the complex L-B is sufficient to recruit FtsW and PBP3. Expression of ZapA-FtsW results in efficient localization of PBP3 in cells depleted of FtsA or FtsQ. Targeting of the complex FtsW-PBP3 restores localization of the FtsQ-L-B complex in cells depleted of FtsA. Thus the late proteins appear capable of associating into pre-assembled complexes (FtsQ-FtsB-FtsL and FtsW-PBP3) within the cell [98]. As the average number of FtsQ proteins in the cell is approximatively 20-40 and that of PBP3 ~100, limited number of protein sub-complexes can be expected along the Z-ring.

FtsW has 10 transmembrane segments (TMSs) and shares 30% identity with RodA and SpoVE, which are involved in elongation and in *Bacillus* sporulation respectively. These proteins appear to work in coordination with one class B PBP to catalyze peptidoglycan polymerization during the cell cycle (see also section 2). The periplasmic loop of FtsW from residue P368 to P375 plays an important role in the septal recruitment of PBP3, the E240-A249 periplasmic amphiphilic sequence appears to be a key element of the functioning of FtsW in the septal peptidoglycan assembly machineries [59]. The first 75 amino acid residues of FtsW are sufficient to interact with FtsQ [90].

The multimodular class B **PBP3** that is specifically involved in septal peptidoglycan synthesis consists of a short intracellular M1-R23 peptide fused to a F24-L39 membrane anchor that is linked via a G40-S70 peptide to the non-catalytic module itself linked to the penicillin-binding module with transpeptidase activity [99, 100]. The first 56 amino acid residues of PBP3 possess the structural determinants required to target the protein to the cell division site, and none of the putative protein interacting peptides present in the N-terminal non-catalytic module are essential for the positioning of the protein at the division site [94]. By using an *E. coli* two-hybrid system, the first 56 amino acid residues of PBP3 were shown to interact with FtsW (unpublished data M. Distèche). Residues 1 to 70 of PBP3 are sufficient to interact with FtsQ, whereas the membrane bound non-catalytic module appears to interact with FtsL [92].

PBP1B localizes at the division site and in the cylindrical part of the cell and interacts directly with PBP3 as shown by using affinity chromatography, surface plasma resonance, two-hybrid system and immunoprecipitation [101]. Its localization depends on the physical presence of PBP3 but not on its activity. A monofunctionalglycosyltransferase (MGT) is able to interact with three constituents of the divisome, PBP3, FtsW and FtsN in a bacterial two-hybrid assay (unpublished M. Terrak and M. Distèche). Finally, PBP1C interacts with PBP3 and PBP1B [102]and the structural protein MipA interacts as well with PBP1B as with the hydrolase MltA [103] (see this issue x). The localization of these proteins is not yet known.

FtsN is a bitopic protein with an N-terminal intracellular domain, a TMS and a periplasmic domain. This region is formed of three short helices (residues 62-123), a long glutamine-rich, unstructured region (residues 124-242) and a C-terminal domain which

binds peptidoglycan but is not essential for cell division [104]. The N-terminal region (residues 1-62) of FtsN although normally not essential for the protein function can compensate for a complete lack of FtsK [105].

FtsN interacts with PBP3 and PBP1B and it stimulates PBP1B activity, indicating that it might have a role in the control of the peptidoglycan synthesis of the third stage of pole synthesis (unpublished Vollmer and Nguyen-Distèche and see section 4.1). A fusion of the subdomain 1C of FtsA to the *Bacillus subtilis* DivIVA protein is targeted to the cell pole and is able to recruit FtsN and PBP3 to the poles independently from FtsZ [106]. FtsN is not recruited by prematurely targeted protein complexes FtsQLB or FtsW-PBP3 and required the presence of FtsA at the division site [98]. FtsA mutants that increase the integrity of the Z-ring can compensate for the loss of FtsN and can partially compensate for a deletion of FtsK or ZipA [107]. FtsN could play a role in regulating conformational changes within the divisome subassemblies, which in turn regulate the activity of the Z-ring. FtsA appears to be conformationally flexible and could be a key modulator of the divisome function at all stages [107].

AmiC is a periplasmic amidase that cleaves the septal peptidoglycan and promotes cell separation. It depends on the presence of FtsN for it localization and it contains a N-terminal domain that is necessary and sufficient to target the protein at the division site [108, 109] (see section 4.1). **EnvC**, a periplasmic metallo-endopeptidase also participates to the splitting of peptidoglycan septum and is located at the division site [110].

4. Rate and topology of peptidoglycan synthesis during the cell cycle

Generation and maintenance of proper morphology in rod shaped bacteria requires a mechanism able to produce a regular cylindrical surface, the lateral peptidoglycan, that grows in length (elongation) whilst avoiding generation of bending and torsional forces. Otherwise growth would result in cells of irregular diameter, with curved or twisting morphologies, shapes often associated with mutational impairment of some morphogenes [1, 111].

At periodical intervals, concomitantly with the onset of cell division, the mode of PG synthesis changes from a constant diameter "elongative" mode to a decreasing diameter "constrictive" mode to form the transversal wall at the cell centre, which will in turn generate the new cell poles as physical separation of the daughter cells proceeds [11, 112-115]. Both elongation and constriction ultimately rely on the local activity of a set of complexes where precursors, originated in the cytoplasm, become polymerized and covalently incorporated into the meshwork structure of the sacculus causing its expansion. However the mechanisms and proteins involved are different enough as to treat both independently in this discussion.

4.1 Topology of insertion sites in the lateral wall of sacculi

Whether incorporation of new precursors into the sacculus happened at a defined location or in a diffuse way was soon decided in favour of the later alternative. Studies performed in the 60's already were supportive of diffuse incorporation in a relatively large number of sites [116]. Application of high resolution autoradiography lead to some initial

confusion, as some early results were interpreted in favour of zonal growth [117]. However, further technical and experimental improvements confirmed the disperse nature of precursor insertion into the lateral wall by a series of complementary approaches as: i) high resolution autoradiography following incorporation of radioactive precursors [118-122]; ii) evolution of the acceptor-donor radioactivity ratio a parameter that indicates whether radioactively labeled incoming precursors cross-link preferentially with themselves or with preexisting material [123, 124]; iii) distribution of peptidoglycan hydrolytic [123, 125], biosynthetic [126, 127] and morphogenetic [17, 128] activities; iv) analysis of the peptidoglycan segregation pattern by D-amino acid labelling and immunomicroscopy [33, 129]; and v) Ramoplanin affinity labeling of precursors [40] and newly incorporated peptidoglycan [33]. Therefore, that elongation of sacculi occurs by incorporation of precursors in a number of homogeneously distributed sites covering the cylindrical surface of the sacculus seems to be a well established fact, at least for the model rod-like bacteria *E. coli, B. subtilis*, and related species. The number of insertion sites active at any particular time point is likely to be rather small. Although the earlier estimations suggested "200 or more" [116], later estimates reduced figures to 80-100 sites per cell based on the kinetics of insertion [130]. Nonetheless, this figure is likely a considerable over-estimation, in view of the scarcity of some critical components of the elongation machinery as PBP2 of *E. coli* (20-40 molecules per cell) [11, 127, 131]. The real nature of the material in the course of insertion is still under debate, in particular whether new glycan chains are inserted individually [132], as pairs [130] or as triplets [113]. Recent evidence supports an intrinsic ability of peptidoglycan synthases to produce pairs of cross-linked glycan strands[133, 134], therefore supporting the idea that glycan

strands are likely to be inserted into the sacculus as cross-linked pairs, or higher order polymers.

The recent demonstration that proper bacterial morphogenesis depends on the dynamics of cytoskeletal proteins as MreB spirals and FtsZ rings [135] supports the concept of insertion sites as wandering structures that travel around the sacculus whilst promoting synthesis and insertion of peptidogycan strands [17, 33, 111, 127, 128]. Actually, the proposal of insertion sites as dynamic entities is anything but new. It was first proposed more than twenty years ago [130], and was also an intrinsic assumption in the "three for one" model for growth of the murein sacculus [113].

That insertion of new material may happen at any place of the lateral wall with equal probability doesn't imply that new and old material is fully intermixed. As a matter of fact, the (sparse) evidence available rather supports generation of mosaic structures made up of interconnected microdomains of all-new and all-old peptidoglycan [33, 136, 137].

In conclusion, our present view is that insertion of precursors into the lateral wall of the sacculus happens at a limited number of discrete sites, which are highly mobile and distributed over the surface of the cell in an MreB associated pattern.

4.2 Topology of precursor insertion sites in septal-polar peptidoglycan

Cell poles are the product of cell division, and by extension polar peptidoglycan is the product of septation. Division of the sacculus requires a change in the mode of synthesis

of macromolecular peptidoglycan. At periodic intervals insertion of precursors instead of promoting elongation starts to generate either an invagination of progressively smaller diameter, or an inwards growing thick transversal septum in Gram negative and Gram positive bacilli, respectively [11, 113-115, 138]. This change has been associated with activation of zonal, that is highly localized, insertion since the earliest studies on cell wall growth; first as a rationalization of the lytic phenotypes observed in response to antibiotics as penicillin [125, 139], and second by high resolution autoradiography [117, 120-122, 140], and peptidoglycan segregation studies [129, 141]. The later study exploited the ability of a, still uncharacterized, periplasmic LD-transpeptidase activity [142] to mediate the exchange of an externally added D-amino acid (D-cysteine) for the D-ala normally present at the C-terminal position of muropeptides. After isolation of the sacculi, the D-Cys are biotinylated and labeled with biotin recognizing gold-labeled secondary antibodies. When growth of the cells in the presence of D-Cys was followed by a chase for one or two mass doublings in the absence of D-Cys, the number of gold particles on the cell poles was still the same compared to unchased samples, whereas the number of particles in the cylindrical part of the cells was diluted by growth (Fig. 4). From these results it was confirmed that the cell poles are constructed from new peptidoglycan and that they become inert after their synthesis [122, 129].

Polar peptidoglycan has a very long life, and seems to remain unchanged for several generations. Neither synthesis nor degradation of peptidoglycan take place in the poles, which can therefore be considered as stable structures [67]. No chemical or structural features clearly accountable for the high stability of polar peptidoglycan have been detected in sacculi. The relatively minor modifications detected are in general

smaller than variations due to growth rate or state of growth [143-147]. An indication that some modification occurs after synthesis of the new cell poles is that the old cell poles do contain recycled peptidoglycan muropeptides, whereas the newly synthesized poles are devoid of these peptides (unpublished data Oldrich, den Blaauwen et al.,). Deficiencies in PBP5, the main DD-carboxypeptidase activity of *E. coli*, have a dramatic influence on the topology of inert peptidoglycan, in particular when combined with mutations on other low molecular weight PBPs as PBP7 [148, 149]. These strains often show morphological alterations as branches, buds and kinks which are always and every time associated to regions of inert peptidoglycan [150]. How these ectopic regions are generated is under study, but evidence indicates interference with normal septation as aberrant poles are a common feature of such strains [150] (unpublished data M.A. de Pedro). An alternative possibility is that dynamics of cytoskeletal proteins preclude biosynthetic complexes from polar regions, as suggested by the influence of FtsZ on peptidoglycan synthesis [52, 111], and in particular on the extension of polar inert peptidoglycan regions [33]. Nevertheless, experimental evidence is insufficient to actually settle the issue.

The detailed architecture and distribution of insertion sites involved in septal peptidoglycan synthesis are still poorly known. Activation of septal peptidoglycan synthesis seems to be a multistep process both regarding assembly of division complexes [80] and insertion of precursors into the central region of the sacculus [121]. Septal synthesis can be divided into at least two stages related to the involvement of cell division proteins; an early stage which requires FtsZ as the only division specific protein, and a later stage which requires the full complement of division proteins [129]. Activation of zonal insertion at the potential division site requires assembly of the FtsZ

ring at the cell centre, but can proceed for a significant period of time in the absence of later division proteins as FtsQ and FtsI, leading to the synthesis of an annulus of all-new murein at the potential division site (Fig. 4).

Once a particular, still unknown, check-point is reached in FtsQ and FtsI filaments, septal synthesis apparently stops and the peptidoglycan biosynthetic potential becomes redirected towards lateral wall growth leaving behind and annulus of inert peptidoglycan [129]. This early step is likely to represent the PIPS (penicillin insensitive peptidoglycan synthesis) stage as proposed by Nanninga [121, 151] on the basis of observations indicative of a penicillin insensitive nature for the initial phase of cell division. When division is allowed to proceed, septal synthesis goes on promoting the progressive constriction of the sacculus. Incorporation of precursors on this phase seems to take place exclusively at the leading edge of the inwards growing constriction, in a PBP3 dependent manner [121]. The nature of the leading edge is still unknown, however evidence from *E. coli* mutants defective in N-acetyl-muramyl-L-alanine amidases supports the idea of the leading edge as a differentiated domain in septal peptidoglycan. N-acetyl-muramyl-L-alanine amidases are required at the terminal stages of septation [145, 152]. Defective *ami*ABC mutants, form chains of cells separated by incomplete septa [109, 152, 153]. Electron microscopy analysis of sacculi from such strains revealed the existence of an electron dense ring structure (Septal Peptidoglycan or SP-ring) tagging the inwards edge of partially constricted division sites [109, 153, 154] (Fig. 5). Furthermore SP-rings are associated to areas of inert peptidoglycan, suggesting that polar peptidoglycan becomes metabolically inert since the very moment of insertion [154]. Apparently SP-rings are able to progress down to a minimum diameter in *ami*ABC

mutants, and then stall. Cytokinesis, however, proceeds to the end and cells become independent entities separated by the respective cytoplasmic membranes [152, 153]. Therefore a potential third (late) stage in septation could be marked by the specific requirement for N-acetyl-muramyl-L-alanine amidases activities to allow the closure of the inwards growing septum and terminate synthesis of polar peptidoglycan.

4.3. Rate of PG synthesis

The rate of peptidoglycan synthesis has been investigated by pulsed incorporation of radioactive peptidoglycan precursors in synchronous growing cells and asynchronous growing cells [121, 155] (see also 4.1). In both cultures the rate of peptidoglycan synthesis was more or less exponential. However when the incorporation of radioactive *meso*-diaminopimelic acid (A_2pm, see this issue ...) was visualized by autoradiography and electron microscopy, an increase in the rate of peptidoglycan synthesis at the constriction site at the expense of a reduction of 40% in the rate of peptidoglycan synthesis in the cylindrical part of the cell was observed [119, 121]. The incorporated radioactive A_2pm was visible after autoradiography as black grains on the cell. In the cylindrical part of the cells the grains were dispersedly distributed whereas they were clustered at the site of division. Even in cells that showed no visible constriction, but that were about to initiate division an increase in the number of grains at mid cell was observed. This increase in mid cell peptidoglycan synthesis is dependent on the presence of FtsZ [119] and coincides with the moment at which the Z-ring is assembled [156]. Remarkably, the increase in mid cell peptidoglycan synthesis is not dependent on the

presence or the function of PBP3 [121] or probably any of the cell division proteins that are recruited to the Z-ring in the second step of divisome maturation [33, 80]. As a consequence, these proteins cannot cause the increased rate of peptidoglycan synthesis at mid cell and the accompanying reduction in lateral peptidoglycan synthesis rate. Similar observations have been made with respect to the two modes of peptidoglycan synthesis (Fig 4). The polar mode of peptidoglycan synthesis is dependent on FtsZ, but not on PBP3 or FtsQ [129]. Therefore, it seems acceptable to assume that the polar mode of peptidoglycan synthesis and the increase in the rate of peptidoglycan synthesis coincide in time and space.

The rate of A_2pm incorporation can be almost completely inhibited either by blocking PBP1A/B by their specific inhibitor cefsulodin or by blocking PBP2 and PBP3 simultaneously by mecillinam and cephalexin [157]. This suggests that the PBP1s and the transpeptidases act together in the synthesis of the cell envelope (see also 4.3). Inhibition of PBP2 in synchronized cells reduced the rate of A_2pm incorporation for 60% during the entire cell cycle whereas inhibition of PBP3 by cephalexin reduced the rate of incorporation especially during the constriction period for 35% [157]. This shows that lateral peptidoglycan synthesis continues during the constriction period, albeit at a lower rate as the number of A_2pm grains in the lateral wall was reduced by 40%. Because the PBP2 activity was more or less equally inhibited in non-constricting cells as in constricting cells (Fig. 6), it can be concluded that PBP2 assist in the synthesis of the new cell poles (see also section 5) whereas PBP3 seems to be restricted to new cell pole synthesis.

The average number of autoradiography A_2pm grains at the division site did not diminish in cells that had progressed in polar cap synthesis compared to division initiating cells [121]. These observations suggest that peptidoglycan synthesis at the leading edge of the constriction site occurs by more or less the same set of peptidoglycan synthesizing protein complexes as those that were recruited during the initiation of cell division. These proteins complexes have to become more clustered during the constriction process when the FtsZ-ring diminishes in diameter. As a consequence of the constant rate of polar peptidoglycan synthesis, the Z-ring diameter decreases with a rate to the power of two. This is in agreement with the relative small fraction of deeply constricting cells in a population.

4.4. Are specific proteins responsible for the local insertion and increase in PG synthesis at the initiation of cell division?

The local increase in peptidoglycan synthesis is dependent on the presence of FtsZ as was discussed above, but not on the proteins that assemble onto the ring in a later stage [80]. It is accompanied by a lengthening of the glycan stands [144] and the synthesis of a band of inert peptidoglycan at the future site of division [129]. Curiously this initiation of constriction, which coincided with Z-ring assembly, could not be inhibited by penicillins such as cefsoludin, mecillinam, furazlocilin, and cephalexin and moenomycin [157]. The presence of a penicillin insensitive peptidoglycan synthetase (PIPS) that interacts with FtsZ was postulated [140] to be responsible for the initiation of polar peptidoglycan synthesis. Could any of the other known peptidoglycan synthesizing proteins be part of PIPS? The third class A PBP of *Escherichia coli*, PBP1C, is

insensitive to the majority of the penicillins and after immobilization on sepharose it was able to retain PBP1B, PBP3, and MltA [102]. It could also retain PBP2 provided that the periplasmic fraction was added to the solubilised membranes [102, 103, 158]. This suggests that PBP1C is at least part of the complex responsible for polar peptidoglycan synthesis and that it could represent PIPS. Because PBP1C is reported to bind to moenomycin coupled agarose it cannot completely represent PIPs. The monofunctional glycosyl transferase (Mgt) of *E. coli* is insensitive to moenomycin [159] and interacts in a bacterial two hybrid system with PBP3, FtsW and FtsN (unpublished data Distèche, Terrak et al.,). The combination of PBP1C and Mgt could provide all the enzymatic activities for PIPS. However, deletion of either or both proteins simultaneously has no effect on the morphology of the cells [102]. The penicillin insensitive LD-transpeptidase of E. coli [142] seems an unlikely candidate as it was shown to be involved in the attachment of the Braun lipoproteine [160]. Since PBP2 is involved in cell pole synthesis [127], it is possible that PBP2 is able to initiate cell division when either PBP3 or PBP1A/B are inhibited. If this would be the case, the peptidoglycan synthetases do not provide any specificity to explain the switch from lateral to polar peptidoglycan synthesis. Specificity would have to be found in, for instance, the activity of the hydrolases such as the carboxypeptidases or in the compartmentalization that the cytoskeleton of the FtsZ-ring and the MreB helix somehow seems to provide. The occurrence of a switch in the mode of peptidoglycan synthesis that leads to new cell pole synthesis is firmly established, but its nature, as is outlined above, is very poorly understood. Future studies might need to include not only the proteins involved in the synthesis of the peptidoglycan layer, but also the physical arrangement of the

biosynthetic complexes as function of the cell cycle and the metabolic and signaling state of the cells.

5. Is the cellular localization of PBPs related to their function?

The PBPs (see also this issue …) are responsible for the periplasmic stage of peptidoglycan synthesis by inserting the lipid II peptidoglycan precursors in the existing peptidoglycan layer during length growth or by synthesizing new peptidoglycan during cell division (section 4). Although some of the PBPs have a unique function, many are redundantly present in most bacterial species investigated thus far. Their specific function, apart from their enzymatic activity, is not very well known. The exceptions are the class B high molecular weight PBPs that have transpeptidase activity. In *E. coli* these are PBP2 and PBP3, which could easily be characterized as essential for length growth and division [11, 161, 162] because their inhibition has such a dramatic effect on cell morphology (i.e. conversion to spheres or filaments, respectively). Deletion of the genes encoding for PBPs has not provided clear-cut information on their function, as PBPs of the same class are often capable of replacing the deleted one. The class A PBP1A and PBP1B in *E. coli* that have transglycosylase as well as transpeptidase activity can each, but not simultaneously, be deleted without any morphological consequences and are apparently not that specific [163]. Surprisingly, all of the four class A PBPs (PBP1, PBP2c, PBP2d, and PBP4) of *B. subtilis* can be simultaneously deleted resulting only in a reduction of growth rate and minor morphological effects [164]. Analysis of the

muropeptide composition of the quadruple-class A mutant strain indicates that a novel unidentified enzyme must perform the glycosyltransferase activity required for peptidoglycan synthesis [164].

An alternative approach to elucidate the function of the various PBPs is to determine where they localize in the bacterial cell using either fluorescent protein fusions or antibodies, and fluorescence microscopy. Promising results have been obtained over the last few years (see for an comprehensive list of localized PBPs; [165]). Localization of PBPs has been studied most extensively in *B. subtilis* were 13 out of the 16 PBPs where localized using strains in which chromosomally encoded PBPs were replaced by GFP-PBP fusions [126, 166]. Three localization patterns could be observed: (i) localization in the cylindrical envelope and at the septum, (ii) septal localization only, and (iii) localization in form of a collection of foci that might be organized in arc-like or helical structures [126]. The last pattern belonged to PBPs that were either lowly expressed or presumably needed for specific growth or sporulation (PBP3, PBP4a, and PBP4*). The class A PBP1B and the class B PBP2B localized at the septum. The class A PBP4, the class B PBP2a, and the major carboxypeptidase PBP5 [167] localized as well in the cylindrical wall as at the septum.

The for lateral growth responsible class B PBP2 of *E. coli* was also shown to be present in the cylindrical part as well as at the constriction using a functional GFP-PBP2 [127] whereas the class B PBP3 responsible for septal peptidoglycan synthesis localizes specifically at the constriction [168]. In the case of PBP2 it was shown that its localization at the constriction was required to maintain the diameter of the new cell poles [127]. Therefore, it seems likely that the new cell poles of rod shaped bacteria are

synthesized with the lateral mode of PG synthesis as well as the septal mode of synthesis, whereas the cylindrical part is only synthesized in the lateral mode. This hypothesis is supported by the observation that some cocci change their shape or diameter after addition of the, for PBP2 specific, antibiotic mecillinam or deletion of genes encoding for proteins that are part of their putative elongation machinery ([6, 169]; see also this issue Y).

The combination of a class A and a class B PBP is in principle sufficient for bulk cell wall synthesis. Based on the localization studies in *B. subtillis* one could envision two separated PG synthesizing machineries as all ready proposed in the eighties by Higgings and Schockman [170]. One for lateral PG synthesis and one for septal PG synthesis where the latter is assisted by the lateral synthesizing machinery during synthesis of the new cell poles. However the observation that the Class A PBP1B localizes in the lateral wall as well as at the septum, where it directly associates with the class B PBP3 [101, 171], which localizes exclusively at the division site, suggests sooner that a rearrangement of the lateral PG machinery occurs to included PBPs such as PBP3 that are specialized in septal PG synthesis. If this would indeed be the case, the, for PBP1B inter exchangeable, PBP1A is expected to localize as well at the constriction site.

The Low Molecular Weight PBPs modify the peptidoglycan by making the peptide side chains inaccessible for cross-linking or by cleaving crosslinks (see this issue X). The morphological defects of deletion of different combinations of the LMW PBPs of *E. coli* produces cells with random shapes, length and diameters [171, 172]. Therefore, the majority of these PBPs are presumably important for fine-tuning of the PG synthesis [173]. Especially the DD-caboxypeptidase PBP5 of *E. coli* seems to be important for the

maintenance of cell diameter, surface uniformity and overall topology of the peptidoglycan layer [149]. Therefore, it is not surprising that *E. coli* PBP5 localizes like *B. subtilis* PBP5 in the lateral wall and predominantly at the septum (unpublished data Karczmarek, den Blaauwen et al.,). In other words it seems to be where the highest PG synthesizing activity occurs. This would agree with the high carboxypeptidase activity reported during division in synchronized cell cultures [174, 175]. In contrast, the carboxypeptidase PBP3 of *Streptococcus pneumoniae* localizes everywhere but at the site of septal peptidoglycan synthesis from which was concluded that by making the peptide side chains of the glycan strands unavailable for crosslinking, the synthesizing PBPs would not be able to localize and insert new cell wall material ([176] and this issue X). Interestingly, overproduction of PBP5 in *E. coli* results in spherical growth [177], which suggests that PBP2 is selective for pentapeptides as peptide cross bridge donors. Possibly, the number of PBP5 molecules of *E. coli and B. subtilis* determines the substrate availability of PBP2 during lateral growth and constriction/septation. Elevated levels of PBP5 or 6 can restore cell division in *ftsI23*(Ts) mutants at the restrictive temperature. Larger amounts of DD-carboxypeptidase increase the amount of tetrapeptide and tripeptide acceptors preferentially used by PBP3. A similar correction of the mutation can be performed by the addition at low concentration of D-cycloserine, which inhibits the D-Ala-D-Ala ligase and thus increases the amount of tripeptide. The level of LD-carboxypeptidase that hydrolyses the tetrapeptide into tripeptide fluctuates during the cell cycle with a sharp increase at the time of division [178]. The proportion of penta-, tetra- and tripeptide might thus be an important factor in determining the cell shape by controlling the relative rate of division and elongation [99].

6. Attachment of the outer membrane to peptidoglycan and consequences for elongation and division

6.1. Linkage between the outer membrane and the petidoglycan

The peptidoglycan layer is connected to the outer membrane via a number of proteins. Of these, the Braun's lipoprotein (Lpp) is the only known protein that is covalently bound to the peptidoglycan while inserted in the outer membrane by its acyl chain [179]. Lpp is the most abundant outer membrane protein and exists in a free (60-70 %) and to peptidoglycan bound (30-40%) form [180, 181]. The L,D transpeptidase YbiS, and to a lesser extent the proteins ErfK and YcfS, are responsible for the linkage of the α-carbonyl of A_2pm to the side chain amide of the L-Lys residue located at the C-terminus of Lpp [160]. The free form might stabilize the peptidoglycan bound form by forming trimers [182]. Lpp is homogenously distributed along the cell envelope [183, 184] and by its sheer numbers, 100,000 copies per μm^2[185], it is in general essential for the maintenance of contacts between the outer membrane and the peptidoglycan layer.

Other protein like the peptidoglycan-associated lipoprotein (PAL) and outer membrane proteins A, C, and F (OmpA, OmpC, and OmpF) interact non-covalently with the sacculus [186, 187]. Deletion of either Lpp or Pal causes loss in the integrity of the outer membrane, release of periplasmic proteins and outer membrane vesicle formation or blebbing [188, 189]. Overexpression of Pal can compensate for the loss of Lpp in an *lpp* deletion strain, but overexpression of Lpp cannot rescue a *pal* deletion strain [190], suggesting that Pal has a more specific function than Lpp.

Pal is anchored to the outer membrane by a N-diacyl glyceride moiety at its second serine residue [191, 192] and interacts strongly with the peptide moiety of the peptidoglycan layer by its carboxy-terminal region [193, 194]). TolB competes with the peptidoglycan peptide side chain for the same region on Pal [195] TolB is part of the Tol-Pal system that connects the inner membrane with the outer membrane. It consists of at least the cytoplasmic membrane proteins TolA, TolQ, and TolR that interact by their transmembrane helices [196], and the periplasmic TolB and Pal protein anchored to the outer membrane. TolQ is an integral membrane protein with three membrane helices whereas TolR and TolA are bitopic membrane proteins with two domains located in the periplasm (see for a review [197]). The C-terminal domain of TolA interacts with the N terminal domain of TonB [198, 199]. The crystal structure of TolB reveals a two-domain structure [200, 201] with a mixed α helix plus sheet of 5 β strands N-terminal domain connected to a six-bladed β-propeller C-terminal domain. This propeller domain interacts with Pal. In addition to the interaction of Pal with TolB and peptidoglycan, it interacts independently with OmpA and with TolA [202]. The interaction of TolA with Pal is lost after dissipation of the electrochemical gradient of ions or protons [202, 203]. Based on sequence comparisons and mutagenesis studies, TolQR have been proposed to form a proton or ion channel, which converts the proton motive force (pmf) to mechanical energy to drive a conformational change in the TolA protein [204]. The channel is probably formed by 14 transmembrane helices derived from a 4:2 stoichiometry of TolQ to TolR [205]. The C-terminal periplasmic domain of TolR seems to have a role in the regulation of the opening and closing of the channel entrance [206]. Whether it regulates

the ion flow by forming a penetrating loop, a plug or a surface structure at the periplasmic side of the channel is not yet clear.

All five proteins of the Tol-Pal system accumulate at the division site during the constriction process and mutants lacking this system exhibit a delay in outer membrane invagination and contain large membrane blebs at the constriction site and the cell poles [207]. The localization of the Tol-Pal at mid cell is dependent on the presence of FtsN and therefore, probably on a mature and functioning divisome. TolA which normally requires FtsN for its recruitment to the divisome, localizes in a *ftsN* null strain suggesting that FtsN does not recruit the Tol-Pal complex directly [107]. Gerding et al., [207] propose that the trans-envelope Tol-Pal system constitutes a subcomplex of the division machinery in Gram-negative bacteria that is specifically employed to ensure proper invagination of the, to the peptidoglycan layer linked, outer membrane. The Tol-pal proteins appear to be uniformly distributed in the bacterial envelope of young cells, to accumulate at mid cell and follow the leading edge of constriction in dividing cells. This dynamic behavior is only possible if the transperiplasmic connection is of a transient nature. The competition of TonB and PG for the same binding site on Pal, and maybe of TolB and TolA for Pal, might allow a discontinuous connection that enables the Pal and Ton proteins to migrate to mid cell. As the same time the connection is sufficiently continuous to force the outer membrane to follow the invaginating cytoplasmic membrane and PG layer and even to be resistant to plasmolysis [208].

The pmf dependence of the Tol-pal interaction might also be related to the frequent occurrence of cell division protein mutants such as FtsZ84(TS) that are sensitive to low or high osmotic strength. Tol-Pal mutant themselves form chains of cells under

osmotic stress conditions [207, 209] indicating that they are deficient in cell division. The cell division proteins FtsE/X have been characterized as conditional osmoremedial essential proteins and deletion of their genes could be compensated for by overproduction of FtsQAZ or FtsN [79]. Reddy [79] suggested that the assembly of the divisome is possibly intrinsically osmosensitive. This osmosensitivity might be caused by the pmf dependence of the Tol-Pal system.

6.2 Mobility of outer membrane proteins is constrained at cell poles

The strong and complex interaction between OM and murein sacculus has a particularly dramatic manifestation in the behaviour of OM proteins at the cell poles. Label and chase experiments designed to follow segregation and mobility of OM proteins showed that proteins at the poles were essentially immobile, whilst in other locations they became progressively intermixed with newly synthesized proteins in the course of cell elongation [210]. In vivo studies on the dynamics of the OM protein LamB also demonstrated coexistence of mobile and static populations of the protein [211]. A likely reason for such unexpected behaviour lays in the inert nature of the peptidoglycan (IPG) making up the polar caps of the sacculus [3]. Since peptidoglycan subunits in the polar regions are locked at their positions, mobility of any other (macro)molecules interacting with these affixed subunits would be constrained. In the case of covalently bound proteins as Braun's lipoprotein [160, 180], or strongly interacting with the sacculus as OmpA [212] or the Tol-Pal complex [207], the proteins become effectively anchored to the subjacent IPG. Because these proteins do interact with the OM, and are present in large numbers, they can in turn affect mobility of other OM proteins and even the physico

chemical properties of the polar area as such. Indeed, differentiation of ectopic or abnormal IPG regions in sacculi of certain mutants is systematically associated to a parallel development of "low protein mobility" areas in the OM [141, 150], thus reinforcing a causal relationship. Whether every single protein species in the OM is similarly affected in the polar area is unknown. Applied methods preferentially labelled a set of proteins accessible to reagents in the extracellular space [210, 211]. Therefore, free movement of non-reacting protein species in and out of the polar regions cannot be excluded. Polar IPG regions might not only influence OM protein mobility but also secretion. Fluorescently labelled proteins in the poles show a very slow decline in absolute signal intensity with time, indicating a similarly slow rate of dilution with newly made, unlabeled proteins [210]. Although still a controversial matter [213, 214], diffusion of periplasmic materials could also be influenced by the temporal stability of the OM-sacculus complex in the polar regions.

The constraint of OM protein mobility at the cell poles is likely to play important roles in a number of cellular functions, as the generation of asymmetries in the distribution of cell envelope proteins [215], polar targeting of specific proteins as IcsA [216, 217], secretion of autotransported proteins [218], twitching mobility [219] and possibly others.

7. L-forms

The definition of what should be called an L-form has been discussed since 1939, when it was shown that many bacterial species gave rise to forms similar to the L1 culture (L

from Lister Institute) that Klieneberger had isolated from *Streptobacillus moniliformis*. Since 1942 the methodology for establishing L-forms routinely involves numerous passages on complex hypertonic penicillin plates over an extended period of time. The first growing cells obtained, the unstable L-forms, are spherical and osmosensitive, and they revert to normal morphology in the absence of penicillin. After further passages, often extending over several years, stable (non-reverting) derivatives are obtained, and some authors have suggested that only these should be called L-forms [220]. Others, however, have presented convincing evidence that stabilization is a secondary event, which simply prevents the reconstitution of a normal cell wall in the absence of penicillin but does not affect L-form growth [221]. What then is an L-form? In the absence of a recognized authority empowered to establish such definitions, the wisest course is to describe clearly the origin and cultivation of the organisms used, whatever name they go by. This, unfortunately, is not always the case in the L-form literature. To avoid confusion, we will call the cefsulodine or penicillin-induced spherical and osmosensitive forms, as "L-form-like" cells.

Because of the prolonged growth of the L-forms in the presence of penicillin and their mycoplasma-like morphology, they are thought to be devoid of cell wall. Electron microscopy showed in some cases that there is no visible cell wall in L-forms of various bacterial species, including *E. coli* [222]. Biochemical analyses of cell wall constituents in L-forms have given variable results, with numerous reports in which muramic acid, A_2pm, D-glutamate, or glucosamine was or was not detected in extracts of L-forms of various bacteria, usually with little quantification [222-224]. So, we are unaware of any

published data that eliminate the possibility of a residual peptidoglycan in an established L-form.

D'Ari and coworkers [225] have found that the growth of L-form-like *E. coli cells* induced by cefsulodin or penicillin, requires residual peptidoglycan synthesis amounting, in the former case, to 7% of that of wild type cells. This is in some ways reminiscent of the paradox of chlamydial species, which are reputed to have no cell wall yet seem to require peptidoglycan synthesis, probably for cell division [226]. The amount of peptidoglycan in L-form-like cells is far too little to form a sacculus covering the entire cell [227]. Although techniques are not presently available for locating this peptidoglycan within the cell, the following arguments suggest that it may be at the division site of the spherical L-form-like cells. When, the for the septation process specific, PBP3 is inhibited by piperacillin in cefsulodin-induced L-form-like cells, a rapid block of the viable cell count is observed. This suggests that PBP3 is required for the propagation of the L-form-Like cells. The transglycosylase activity of PBP1B is required for rapid growth of the L-form-like cells, and this protein has been shown to interact directly with PBP3 [101]. In addition, PBP1B has been implicated in cell division under certain conditions [228]. The division protein FtsZ is also required for the propagation of L-form-like cells [225]. The simplest hypothesis to explain these observations – requirements for peptidoglycan synthesis, FtsZ, PBP3, and PBP1B – is that cell division in cefsulodin-induced L-form-like cells, as in rods, by means of peptidoglycan synthesized in the division plane and indispensable for cytokinesis [225].

It was recently reported that an established *E. coli* L-form isolated nearly 40 years ago has acquired mutations in several genes required for peptidoglycan synthesis and cell

division [229]. From the sequence of 36 kb of L-form DNA, the authors deduced that the FtsA, FtsW and MurG proteins have one or two amino acid changes each, the *ftsQ* gene has an amber triplet at codon 132 (of 276 codons), and the *mraY* gene has a frame shift in codon 294 that should produce a protein of 298 amino acids (instead of 360). The functional consequences, in rod-shaped cells, of the missense mutations and of the truncation of MraY are unknown. The truncated FtsQ protein would almost certainly be non-functional for cell division in rod-shaped cells, although a low level of amber suppression could provide the 22 molecules of intact FtsQ estimated to be needed for division [230]. Further characterization of this classical L-form should establish clearly whether or not the cells carry out residual peptidoglycan synthesis and, if they do, whether it is essential for their propagation.

Cell division in normal bacteria is inseparable from cell wall synthesis at the septum, and now [225] it has been shown that this requirement is also present for L-form-like cells. The question is why cell division would require peptidoglycan synthesis, and the simplest answer is that macromolecular PG is both substrate and product of the reaction, and the residual 7% of macromolecular PG would provide the required scaffold for assembly of division leading edge.

Concluding remarks

The elongation of the sacculus is performed by a dynamic protein complex “the elongase” that inserts peptidoglycan precursors at a limited number of discrete sites while using the cytoskeletal MreB helix as tracking devise. The elongase consists of at least; MraY, MurG, MreBCD, RodA, PBP1, and PBP2. Based on the average number of MurG

foci in the cylindrical part of the cell [46]and the number of PBP2 molecules per average cell [231], it seems unrealistic to expect more than 50 elongases per average cell depending on the growth rate (Fig 9). Upon initiation of cell division by positioning of the cytoskeletal Z-ring at mid cell, a switch from the dispersed to a concentrated local peptidoglycan synthesis occurs. From this point on peptidoglycan synthesis is for a large part redirected from the elongating activity to the synthesis of new cell poles that consists of new peptidoglycan. This synthesis is performed by the divisome or septosome. Initially the synthesis seems to be performed by a premature divisome that contains PIPS (see section 4.4) to synthesize new peptidoglycan. After about one fifth of a generation time the divisome matures and might be envisioned as an extended elongase because apart from its basic peptidoglycan synthesis activity specific functions have to be added. These are, the change in morphology from cylinder to sphere (e.g. FtsK, FtsQLB, FtsW, PBP3), the invagination of the outer membrane (e.g. Tol-Pal-FtsN), and the hydrolases that allow the extension and invagination of the peptidoglycan layer (e.g. MipA, MltA, AmiA, EnvA). Again, beased on the number of tightly membrane bound FtsZ molecules (unpublished data den Blaauwen) and the FtsQ, PBP3 molecules present per average cell [90, 231], not more than 50 divisome subassemblies will be present at the division site (Fig. 9). As the rate of peptidogycan synthesis at the leading edge is constant during cell division, these subassemblies will be active during the progression of the leading edge to the closure of the constriction into a separated daughter cell pole (Fig. 9). The elongase and the divisome are dynamic hyperstructures [232] that probably at least share part of their proteins (e.g. PBP1, PBP2, MurG, MraY?, carboxypeptidases). A picture is emerging in which the PBPs, on which the focus have been for the major part of the last

century, are the proteins that are the least involved in the regulation of the creation of the shape maintaining sacculus.

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